

REMARKS

Status of the Claims

Claims 1, 2 and 4-21 are currently pending in the application. Claims 1-5, 18 and 19 stand rejected. Claims 6-17 and 20 are withdrawn as being drawn to a non-elected invention. Claims 1, 2, 4 and 18 have been amended as set forth herein. Claim 3 has been cancelled herein. All amendments and cancellations are made without prejudice or disclaimer. New claim 21 is presented herein. No new matter has been added by way of the present amendments. Specifically, the amendment to claim 1 is supported by the specification at, for instance, page 2, lines 8-10, page 3, and Examples 1 and 2. Amendment to claims 2 and 18 are supported throughout the specification at, for instance, page 10, last paragraph. Claim 4 has been amended herein to depend from both claim 1 and claim 2. New claim 21 is supported at, for instance, claims 1, 2 and 5. Reconsideration is respectfully requested.

Objections to the Specification

The Examiner objects to the specification for reciting trademarks without capitalization. (See, Office Action of January 17, 2007, at page 3, hereinafter, "Office Action"). Applicants have submitted herewith amendments to the specification correcting these errors. In the amendments, the entire trademark name is capitalized. No new matter is introduced to the specification by way of these amendments.

Reconsideration and withdrawal of the objection to the specification are respectfully requested.

Rejections Under 35 U.S.C. § 112, Second Paragraph

Claims 1-3, 18 and 19 stand rejected under 35 U.S.C. § 112, second paragraph, for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. (See, Office Action, at page 3). Claim 3 has been cancelled herein without prejudice or disclaimer, thus obviating the rejection of claim 3. Applicants traverse the rejection as to the remaining claims as set forth herein.

The Examiner states that the phrase "biological activity substantially equivalent to the function of the polypeptide," recited in claims 1 and 2 is unclear because this phrase could be interpreted to mean many different things and clarification by amendment of the claims is required. (*Id.* at pages 3-4).

Although Applicants do not agree that claims 1 and 2 are indefinite, to expedite prosecution, claims 1 and 2 have been amended herein without prejudice or disclaimer to remove the phrase upon which the Examiner's rejection is based. In place thereof Applicants have amended claims 1 and 2 to recite, in part, "having N-acetylglucosamine transferase activity." Support for this amendment may be found throughout the specification at, for instance, page 2, lines 8-10, page 3, and Examples 1 and 2. That is, as shown in Examples 1 and 2, the isolated DNA of the present invention is clone number FJ04470. The specification, at page 2, lines 8-10, page 3 and Example 2, shows that the protein encoded by clone FJ04470 has N-acetylglucosamine transferase activity, thus providing basis for support for the amendment. Furthermore, Applicants submit herewith a copy of Inamori et al., *The Journal of Biological Chemistry*, 278(44):43102-43109, 2003, attached hereto as Exhibit A, which was published after the priority date of the present application. Inamori et al. disclose a protein (GnT-IX) which has

an amino acid sequence 99.9% identical to SEQ ID NO:2 of the present application. Also attached hereto as Exhibit B is a Figure showing the alignment of the amino acid sequence of Inamori et al. (Q76D04 protein) with the amino acid sequence of SEQ ID NO:2 of the present application (X2375 FJ04470, SEQ ID NO:2). As the Figure in Exhibit B indicates, only one amino acid is different between the two sequences. Inamori et al. further show that a recombinant GnT-IX has N-acetylglucosamine transferase activity, as disclosed in the abstract and in the second paragraph in the right column on page 43108. Therefore, it is clear that the protein encoded by the claimed DNA of the present application has N-acetylglucosamine transferase activity.

The Examiner further states that the recitation of "stringent conditions" of hybridization of claims 2 and 18 is unclear. (*Id.* at page 4). Although Applicants do not agree that claims 2 and 18 are indefinite, to expedite prosecution, claims 2 and 18 have been amended herein without prejudice or disclaimer to recite, in part, "hybridizing under stringent conditions of 0.1 XSSC, 0.1% SDS and 37 °C." Support for this amendment may be found throughout the specification at, for instance, page 10, last paragraph.

Reconsideration and withdrawal of the indefiniteness rejection of claims 1, 2, 18 and 19 are respectfully requested.

Rejections Under 35 U.S.C. § 112, First Paragraph

Claims 1-5, 18 and 19 stand rejected under 35 U.S.C. § 112, first paragraph, for failing to comply with the enablement requirement. (See, Office Action, at page 4). Claim 3 has been

cancelled herein without prejudice or disclaimer, thus obviating the rejection of claim 3. Applicants traverse the rejection as to the remaining claims as set forth herein.

The Examiner states that the specification enables a DNA encoding SEQ ID NO:2 or a DNA comprising SEQ ID NO:1. (*Id.* at page 5). However, the Examiner states that the specification does not enable any DNA sequences having any deletions, substitutions or additions to these sequences or nucleotides that hybridize to these sequences, complementary sequences or genes comprising these sequences. (*Id.*). The Examiner's comments seem to be directed at the "derived from" language of claims 1 and 2 and the language including the phrase "deletion, substitution, or addition of one or a plurality of amino acids." (*Id.*).

Although Applicants do not agree that claims 1, 2, 4, 5, 18 and 19 lack enablement in the specification, claim 1, part (b), has been amended herein, without prejudice or disclaimer, to recite, in part, "a polypeptide, consisting of an amino acid sequence derived from the amino acid sequence represented by SEQ ID NO: 2 by deletion, substitution, or addition of within one to twenty amino acids and having N-acetylglucosamine transferase activity." This amendment is supported by the specification at, for instance, the second paragraph of page 9. The number of amino acids of the sequence of SEQ ID NO:2 is 793. The amino acid sequence derived from the amino acid sequence represented by SEQ ID NO: 2 by deletion, substitution, or addition of within one to twenty amino acids has more than 95% identity with the amino acid sequence of SEQ ID NO:2. Accordingly, those skilled in the art can readily obtain a polypeptide consisting of an amino acid sequence derived from the amino acid sequence represented by SEQ ID NO: 2 by deletion, substitution, or addition of within one to twenty amino acids and having N-acetylglucosamine transferase activity.

Regarding claims 2 and 18, although Applicants do not agree that these claims lack enablement, to expedite prosecution, claims 2 and 18 have been amended without prejudice or disclaimer to recite, in part, "hybridizing under stringent conditions of 0.1 XSSC, 0.1% SDS and 37 °C." Support for this amendment may be found throughout the specification at, for instance, page 10, last paragraph.

Since no independent reasoning is provided for the rejection of dependent claims 4, 5 and 19, these claims are believed to also be enabled for the reasons provided above, with respect to claims 1, 2 and 18, from which they depend.

Reconsideration and withdrawal of the enablement rejection of claims 1, 2, 4, 5, 18 and 19 are respectfully requested.

Rejections Under 35 U.S.C. § 101

Claims 1-3, 18 and 19 stand rejected under 35 U.S.C. § 101 because they allegedly are directed to non-statutory subject matter. (See, Office Action, at page 7). Claim 3 has been cancelled herein without prejudice or disclaimer, thus obviating the rejection of claim 3. Applicants traverse the rejection as to the remaining claims as set forth herein.

The Examiner suggests amending the claims to recite "an isolated" or "purified" nucleotide or polypeptide to reflect the hand of man in the presently claimed invention. (*Id.* at page 8). Applicants have herein amended claims 1, 2, 18 and 19 (be dependency) to recite "isolated" polynucleotide or DNA, as suggested by the Examiner.

Reconsideration and withdrawal of the rejection of claims 1, 2, 18 and 19 in light of the amendments presented herein are respectfully requested.

Rejections Under 35 U.S.C. § 102(b)

Claims 1-5 and 18 stand rejected under 35 U.S.C. § 102(b) as being anticipated by Sanjanwala et al., WO 2002/46426 (hereinafter, "Sanjanwala et al."). (See, Office Action, at page 8). Claim 3 has been cancelled herein without prejudice or disclaimer, thus obviating the rejection of claim 3. Applicants traverse the rejection as to the remaining claims as set forth herein.

The Examiner states that Sanjanwala et al. disclose a nucleotide sequence (SEQ ID NO:8) which is 86.5% homologous to SEQ ID NO:2 of the presently claimed invention and that Sanjanwala et al. disclose a gene comprising this sequence as well as a cell expressing the protein. (*Id.* at pages 8-9). The Examiner further states that since it is likely a polynucleotide encoding SEQ ID NO:8 of Sanjanwala et al. would hybridize to SEQ ID NO:1 under some conditions and may encode an enzyme having similar activity, Sanjanwala et al. anticipate the presently claimed invention. (*Id.*).

However, claim 1 has been amended herein to recite that the isolated DNA of claim 1, part (b), encompass SEQ ID NO:2 and those sequences varying by only 1 to 20 amino acids, which is at least 95% identical to SEQ ID NO:2. The sequence disclosed in Sanjawala et al. is only 86.5% identical, as stated by the Examiner at page 9 of the Office Action. Furthermore, claims 2 and 18 have been amended to recite specific stringent hybridization conditions. Sanjawala et al. disclose no sequences that would hybridize to the isolated DNA of the presently claimed invention.

Thus, since the cited reference does not disclose all of the limitations of the presently claimed invention, Sanjawala et al. cannot anticipate the presently claimed invention. "A claim

is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." (See, *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 U.S.P.Q.2d 1051, 1053 (Fed. Cir. 1987)).

Dependent claims 4, 5 and 19 are not anticipated as, *inter alia*, depending from a non-anticipated base claims, claims 1 and 2.

Reconsideration and withdrawal of the anticipation rejection of claims 1, 2, 4, 5, 18 and 19 are respectfully requested.

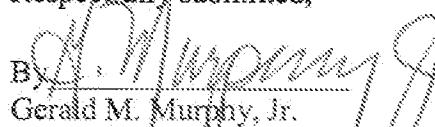
CONCLUSION

If the Examiner has any questions or comments, please contact Thomas J. Siepmann, Ph.D., Registration No 57,374, at the offices of Birch, Stewart, Kolasch & Birch, LLP.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to our Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under § 1.17; particularly, extension of time fees.

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Respectfully submitted,

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Attachments: Exhibit A - Inamori et al., *J. Biol. Chem.*, 278(44):43102-43109, 2003
Exhibit B – Sequence Alignment, Q76D04 (GnT-IX) and SEQ ID NO:2

Molecular Cloning and Characterization of Human GnT-IX, a Novel β 1,6-N-Acetylglucosaminyltransferase That Is Specifically Expressed in the Brain*

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A novel β 1,6-N-acetylglucosaminyltransferase (GnT) cDNA was identified by a BLAST search using the amino acid sequence of human GnT-V as a query. The full-length sequence was determined by a combination of 5'-rapid amplification of cDNA end analysis and a further data base search. The open reading frame encodes a 792 amino acid protein with a type II membrane protein structure typical of glycosyltransferases. The entire sequence identity to human GnT-V is 42%. When pyridylaminated (PA) agalacto biantennary N-linked oligosaccharide was used as an acceptor substrate, the recombinant enzyme generated a novel product other than the expected GnT-V product, (GlcNAc β 1,2-Man α 1,3)[GlcNAc β 1,6-Man α 1,6]Man β 1,4-GlcNAc β 1,4-GlcNAc-PA. This new product was identified as [GlcNAc β 1,2-(GlcNAc β 1,6-Man α 1,3)][GlcNAc β 1,2-(GlcNAc β 1,6-Man α 1,6]Man β 1,4-GlcNAc β 1,4-GlcNAc-PA by mass spectrometry and 1 H NMR. Namely, the new GnT (designated as GnT-IX) has β 1,6GnT activity not only to the α 1,6-linked mannose arm but also to the α 1,3-linked mannose arm of N-glycan, forming a unique structure that has not been reported to date. Northern blot analysis showed that the GnT-IX gene is exclusively expressed in the brain, whereas the GnT-V gene is expressed ubiquitously. These results suggest that GnT-IX is responsible for the synthesis of a unique oligosaccharide structure in the brain.

The biological roles of N-linked oligosaccharides on glycoproteins are thought to play a role in the interaction of terminal glycan structures and their receptors. The diversity and avidity of the terminal structures are, however, regulated by the core structure of N-glycans (1). In vertebrates, six different

GnTs¹ (GnT-I through GnT-VI) are involved in the biosynthesis of the highly branched complex-type N-glycan core structure (1). A β 1,6GnT, GnT-V, catalyzes the transfer of GlcNAc from UDP-GlcNAc to the core α 1,6-mannose arm via a β 1,6-linkage, forming the tri- and tetraantennary complex-type N-glycans (1).

The β 1,6-branched tri- and tetraantennary glycans are preferentially elongated via the action of β 1,3GnT and β 1,4-galactosyltransferase to produce a poly-N-acetyllactosamine structure (2, 3). In addition, the N-glycans in GnT-V-deficient cells are severely depleted of poly-N-acetyllactosamine but not O-glycans (4, 5). Poly-N-acetyllactosamine chains are known to serve as ligands for cell adhesion molecules such as selectins and galectins (6–8). It has been suggested that changes in poly-N-acetyllactosamine content on sympathetic nerve cells affect their migration (9, 10). Furthermore, N-acetyllactosamine promotes the neural outgrowth of primary olfactory neurons (11). These collective findings suggest that GnT-V activity in the brain may play an important role in the process of nerve differentiation via the interaction of poly-N-acetyllactosamine chains and their receptors. However, the GnT-V gene (*Mgat5*)-knockout mice that apparently lack both detectable GnT-V activity and *Phaseolus vulgaris* leukoagglutinin reactivity in the brain, appear to be neurologically normal (12). Since a detailed analysis of the carbohydrate chains of these glycoproteins has not been examined, the issue of whether poly-N-acetyllactosamine structure is, in fact, altered in *Mgat5*^{−/−} brain remains to be elucidated.

When the GnT-V gene was cloned, no homologous genes were evident, suggesting that GnT-V has a unique evolutionary origin (13, 14). The GnT-V ortholog gene (*gly-2*) was recently identified in *Caenorhabditis elegans*, and there is no homologous gene in this organism (15). However, the recent, rapid accumulation of EST and genomic data enabled us to identify a novel human β 1,6GnT gene homologous to GnT-V. This new GnT catalyzes the synthesis of a unique N-linked oligosaccharide structure and was designated as GnT-IX, since two additional GnT activities (GnT-VII and -VIII) were demonstrated in CHO mutant cells, although their genes have not been identified (16). Interestingly, the GnT-IX gene is exclusively ex-

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB109185.

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¹ The abbreviations used are: GnT, N-acetylglucosaminyltransferase; PA, pyridylaminated; EST, expressed sequence tag; KACE, rapid amplification of cDNA ends; GSP, gene-specific primer; Ni-NTA, Ni²⁺-nitrotriacetic acid; MES, 2-morpholinethanesulfonic acid; MOPS, 3-morpholino-2-propanesulfonic acid; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry.

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Gmt-IX MTTVNPDGKIMVRRCLVTLRPFRLPVLGIGFTFLCFLMTSLGGQFSARRIQLDSEFTTFTSVNGSPESPGVIRKNSDILRL 80
 Gmt-V MALTFTP-WKLSSQXLYCPFYLVFG-PWGNMMLHAPTIQORTQPESSSMRREQILBLQKPYIKALEBNRNVVDCPYRGVMT 78
 * * * * *
 Gmt-IX MVKRMDALARIENSGSELHRAQGDIQHFFPADRKMPGQSLMRSRQIAQINQNSDEIAVKVTDQILRNSLLIANSKVEGREGDQCAP 160
 Gmt-V AYDLKKTLAVLLDN-ILORIG-KLESKVDNLLVNG-----TQINSTNSTAVPSLVALEKCNVANIINGAQECKVLP 148
 * * * * *
 Gmt-IX SDFPKFDCEGKVENMPARWTSOPCYAPPGVGDGTECSFLYILSEVENWFCPPLPWRNQTAACQRAPKPLPFWQAVFRSNLNSHL 240
 Gmt-V PMDGYPHCEGRIKRMKMDM-ESDPFCYADYGVUDGTSCTSYTIVLSSVENNWCPHLYWRAKMPYSEADH--WSLASIRTOFNMIL 228
 * * * * *
 Gmt-IX LEIMQSGKESLIFMKRTRKLTQWALIAQRIIAQKLGATQRQIKQKQIINHIGFLTCEEGDQVSPRVLKGGPLHNMVQWADL 328
 Gmt-V YSMRKE-HEEFPRMNLIRKIRRMADAWIAQJKNLAQCNLLEKRRKRRRNVYHLQQLLTKESSGPPIAETAFSGSPICLVLWQNSDL 304
 * * * * *
 Gmt-IX LTALVVLGHGLRVTVSLXRLQSMHGVPPG-PGSCPLUTMPLPFDLITYDQHKLQQMPKXMGCLSFKKYRCPIDV10TPCTEP 399
 Gmt-V ITTSLYLGHGDKRISASLXRLKRMKKVVGNGPSCPTVGDRLVTELTYDQIVGLAQPFKTLGPSPKSVVIVQCNLRLVLDSPGTEP 384
 * * * * *
 Gmt-IX AYKHEEYATLNGCYTNTMCWNLNPXQFTMIFHTPDNSTYMGTEVSE--LNEETKRLIKGRHLSAHSNAYVYVGEASIWKLQK 478
 Gmt-V EFNHARTAQSGKGNKTPWCKWNLNPFQFQFTMIFHTPDNSTFLCFSVVECHNNSDTHNNRINRQDQSLVYKQVSPWNS--K 462
 * * * * *
 Gmt-IX EKPLG-LINKYKHSIHTVYYESQRPFPEVPAFYKHNQHLLPQPEFOQQLRRAKLPIGFSEFPYEGPAFLEAIANGCIFLQERPS 558
 Gmt-V KIVYDILHTYNEVIAITVYGSSTK- IPSYVNNGILSGRDLQFLLRETKLFWVGLGFPYEGPAFLEAIANGCAFLNPKP 540
 * * * * *
 Gmt-IX PPKHESLHHEPFPRGKPTSRQVPQNPYAAENPICKPBNWTFDYNNSSEFBAIKRIMRTQVDPDYLPYETCTEGMLERIRHAYI 838
 Gmt-V PPKHESLHHEPFPRGKPTSRQVPQNPYAAENPICKPBNWTFDYNNSSEFBAIKRIMRTQVDPDYLPYETCTEGMLERIRHAYI 826
 * * * * *
 Gmt-IX QHQDFCRAPDPALFHRHAPQSPPVFLAPNATHLEWANRTELAPSANPPAKALRANLWAVPGRACIDTCIQLH3Q1C1C8PEFFFF 718
 Gmt-V ENQDPCHG- QVWMPPLSALGVKLAEPGQSCQKVQCBSQLICEPSPYFQH 667
 * * * * *
 Gmt-IX LNSQGQANFLKLQVSCDOSTESEHNNHLYFAPAQAGQGKQBLQNEPGLPSCQGNSNTYKRRILCPKRCDFPNSQVRLCQGQH 792
 Gmt-V INKQKSDSLKMYKTYCQSSHLAKDILVPSFDPKHKHUVFQGDLLLPSCAHSHFREQBNVCPICDFEIQVQALCKDCL 741

FIG. 1. Nucleotide and deduced amino acid sequences of human GnT-IX. *A*, the putative transmembrane domain is single underlined. The potential *N*-glycosylation sites are circled. *B*, alignment of the amino acid sequences of human GnT-IX with human GnT-V (14). The asterisks below the sequences indicate identical residues.

pressed in the brain. Thus, GaT-IX may play an essential role in the synthesis of such novel oligosaccharide structures in the brain. In addition, GaT-IX may compensate for the deficiency in *MgaT5^{-/-}* brain (12) with regard to neuronal development and functions.

EXPERIMENTAL PROCEDURES

Materials—A fluorescence-labeled acceptor substrate GnGn-bi-PA, GlcNAc β 1,2-Man α 1,3-(GlcNAc β 1,2-Man α 1,6-)Man β 1,4-GlcNAc β 1,4-GlcNAc-PA was prepared as reported previously (17, 18). Gn α -teti-PA, GlcNAc β 1,2-Man α 1,3-(GlcNAc β 1,2-GlcNAc β 1,6-)Man α 1,6-Man β 1,4-GlcNAc β 1,4-GlcNAc-FA was prepared by a large scale GnT-V reaction

using GcGc-bi-PA as a substrate followed by the isolation of the reaction product using TSKgel ODS-80TM columns (7.8 × 300 mm; TOSOH) as described under "GcT Assay."

cDNA Cloning of GnT-IX—Using the amino acid sequence of human GnT-V (14) as a query for a BLAST search at the DNA Data Bank of Japan homology search system, we found a fragment sequence, human hypothetical protein T50606, that exhibited a significant similarity to GnT-V. Using the amino acid sequence of T50606 for an EST data base search, seven clones (AL524151, BF563340, HE785253, BE789945, R87580, BF038743, and R87617) were identified that contained partial sequences corresponding to T50606. One μ g of human brain total RNA (OriGene Technologies) was reverse-transcribed and then amplified by PCR with a pair of primers designed from the consensus sequence of the

seven EST clones: S1 (5'-AGGGACCAGAAGCAGAT-3', corresponding to nucleotides 841-857 in Fig. 1) and AS1 (5'-GAACCTGCTCGT-GCTTT-3', corresponding to nucleotides 2495-2451). The PCR product

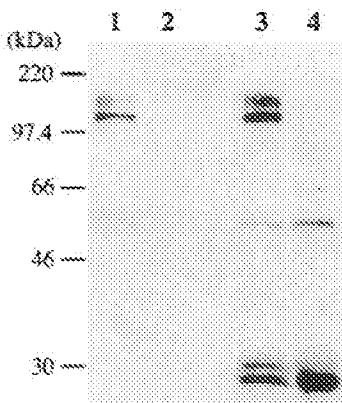


Fig. 2. Western blot analysis of captured proteins by Ni-NTA beads and immunoprecipitates. Ni-NTA-captured proteins (lanes 1 and 2) and immunoprecipitates (lanes 3 and 4) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with an anti-His tag antibody and then incubated with peroxidase-conjugated anti-mouse IgG. Lanes 1 and 3, Neuro-2a/GnT-IX cells; lanes 2 and 4, parental Neuro-2a cells. Standards are indicated on the left.

was subcloned into pETBlue vector (Novagen) and then sequenced by the dideoxy chain termination method using DNA sequencers (Applied Biosystems model 377 and 310). The 5'-end of the GnT-IX was determined by the combination of rapid amplification of cDNA ends (RACE) using a 5'-RACE system kit (Invitrogen). Briefly, 5 μ g of human brain total RNA was reverse-transcribed with the GnT-IX gene-specific antisense primer GSPI (5'-TACTCTGAGCTCTT-3', corresponding to nucleotides 1012-1027), and the first strand cDNA was tailed at the 3'-end by the terminal deoxynucleotidyltransferase with dCTP followed by PCR with 5'-RACE abridged anchor primer (5'-GGCAGCGCGTC-GACTAGTACGGGHHGGGGHGGGGH-3') and GSPI. The PCR product was size-selected (approximately 0.5-1 kb) and then subjected to nested PCR with abridged universal amplification primer (5'-GGCACCGCTGACTAGTAC-3') and GSPI (5'-CCAGGACATAGAGTC-GAGTC-3', corresponding to nucleotides 963-982). The sequence of the PCR product was used as a query for a further EST data base search. After aligning the sequences of the EST clones (BE276066, BE391943, BE730366, and BG478724) containing the putative start codon, the open reading frame was amplified by PCR with a pair of primers: 5'-CTGCTCCACCAACAGT-3', corresponding to nucleotides -25 to -8 and AS1. The PCR product was subcloned and sequenced as described above.

Construction of a Vector Encoding His₆-tagged Human GnT-IX—To prepare a C-terminal His₆-tagged GnT-IX construct, the full-length cDNA and a Myc-His₆ tag sequence were ligated to pcDNA3.1/Zeo(+)(Invitrogen) with KpnI/EcoRV sites. To prepare a soluble N-terminal His₆-tagged version of the construct, GnT-IX cDNA was amplified by PCR with primers 5'-GCGAAATTGACCATCGCACAG-3' and 5'-CCGTCTAGATCACAGACAGCCCTGGC-3' and then subcloned into pcDNA3.1/Zeo with IgG signal and His₆ tag sequences.

Expression of Human GnT-IX in Neuro-2a Cells—Neuro-2a cells

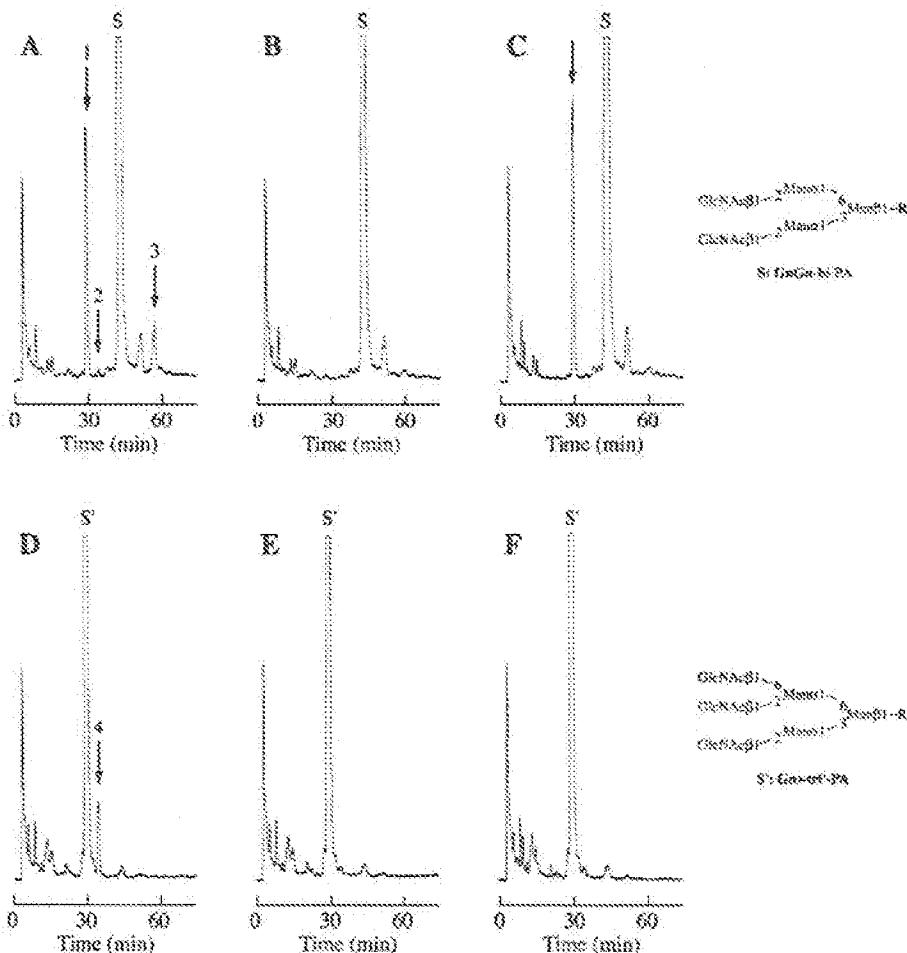


Fig. 3. Elution profile of the reaction mixture on HPLC. A reaction mixture, using GNGn-PA (A-C) or GNGn-tri-PA (D-F) as a substrate (S or S') and captured proteins from Neuro-2a/GnT-IX (A and D) or from parental Neuro-2a cells (B and E) by Ni-NTA beads, or a microsomal fraction of COS-1 cells transfected with pSV2neo-GnT-V (C and F) as an enzyme source was applied to a TSKgel ODS-80TM column and eluted as described under "Experimental Procedures." The arrows indicate the positions of the eluted products.

were transfected with the linearized plasmid using the SuperFect transfection reagent (Qiagen) according to the standard protocol used for stable transfection and selected for clones that stably express the gene, based on resistance to Zeocin (Invitrogen) followed by detection of the expression of the His₆-tagged protein by Western blot analysis with an anti-His tag antibody (Tetra His Antibody; Qiagen). The cloned cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 100 µg/ml Zeocin. The cells expressing full-length GnT-IX were washed and pelleted, and the microsomal fraction was prepared as described previously (19). For the expression of the soluble form of GnT-IX, the cells were cultured for 3 days in the same medium with 1% serum and antibiotics without Zeocin. The soluble His₆-tagged enzyme was partially purified by passing it through a chelating Sepharose Fast Flow (Amersham Biosciences) column according to the manufacturer's instruction. For expression of GnT-V as a positive control for the transferase assay, COS-1 cells were transfected with the pSVK3-hGnT-V vector as described previously (18). The microsomal fraction of the transfected cells was prepared, as described above.

Capture and Immunoprecipitation of His₆-tagged Enzyme—For the capture of the tagged enzyme, the solubilized microsomal fraction was incubated with Ni-NTA Superflow beads (Qiagen) in 20 mM imidazole, 1% Triton X-100, phosphate-buffered saline at 4 °C overnight, and the beads were then washed four times with 20 mM imidazole, 0.5% Triton X-100, phosphate-buffered saline. Immunoprecipitation was performed using an anti-His tag antibody. Briefly, the solubilized microsomal fraction was incubated with 5–10 µg/ml of the antibody for 1 h and then with Protein G-Sepharose 4 Fast Flow (Amersham Biosciences) for 1 h. The immune complex was washed four times with 0.5% Triton X-100, phosphate-buffered saline. The resulting enzyme captured by Ni-NTA beads or immunoprecipitated was subjected to Western blot analysis and a GnT assay.

Western Blot Analysis—The captured His₆-tagged proteins were separated by SDS-PAGE (10% Laemmli's gel) and transferred to a nitrocellulose membrane (Schleicher & Schuell). The membrane was probed with an anti-His tag antibody and then incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG. The reactive proteins were visualized by means of an ECL kit (Amersham Biosciences).

GnT Assay—GnT activity was assayed using pyridylaminated acceptor substrates under conditions described previously (20) with minor modifications. The sample was incubated at 37 °C for 4 h with 20 µM pyridylaminated acceptor substrate (GnGn-bi-PA or Gn₃-tri-PA) and 40 mM UDP-GlcNAc in 125 mM MES (pH 6.25) or MOPS (pH 7.5) containing 200 mM GlcNAc, 0.5% Triton X-100, and 10 mM EDTA. The reaction was terminated by boiling for 3 min and then centrifuged at 15,000 rpm for 5 min. The resulting supernatant was injected to a TSKgel ODS-80TM column (4.6 × 250 mm; TOSOH) equipped with a Shimadzu LC-VP HPLC system. Product was eluted with 0.1 M ammonium acetate (pH 4.0) with or without 0.05% *n*-butyl alcohol at a flow rate of 1.0 ml/min and monitored with a fluorescence spectrophotometer (excitation, 320 nm; emission, 406 nm).

MALDI-TOF MS Analysis—MALDI-TOF MS was performed with a Perseptive Biosystems Voyager RP-DE instrument. The mass spectra were acquired in the reflection mode under a 20-kV accelerating voltage with positive detection. 2,5-dihydroxybenzoic acid (10 mg/ml) was used as the matrix.

NMR Analysis—For preparation of the NMR sample, a large scale reaction was carried out with 500 µM GnGn-bi-PA as a substrate in the presence of 0.5 M glycine. After a 3-day incubation at 37 °C, the reaction product was isolated using a TSKgel ODS-80TM column (7.8 × 300 mm; TOSOH) as described above. The separated sugar chains were lyophilized, dissolved in 99.9% D₂O, and then lyophilized from D₂O twice and dissolved in this solvent. Proton NMR measurements were carried out with a Varian Unity-400 spectrometer at 400 MHz at 30 °C. Chemical shifts are expressed as ppm relative to an external standard of 3-(tri-methylsilyl)propionic acid-d₄.

Northern Blot and Dot Blot Analyses—Human 12-lane multiple tissue Northern blot and human multiple tissue expression array (Clontech) were used for hybridization according to the manufacturer's protocol. Probes were prepared from the cDNA fragments from a PvuII digest (corresponding to nucleotides 1564–2286) of pSVK3-hGnT-IX, pSVK3 vector (Amersham Biosciences) containing human GnT-IX cDNA, and an EcoRV digest (corresponding to nucleotides 406–1306) of pSVK3-hGnT-V and then labeled with [α -³²P]dCTP using a Megaprime DNA labeling system (Amersham Biosciences).

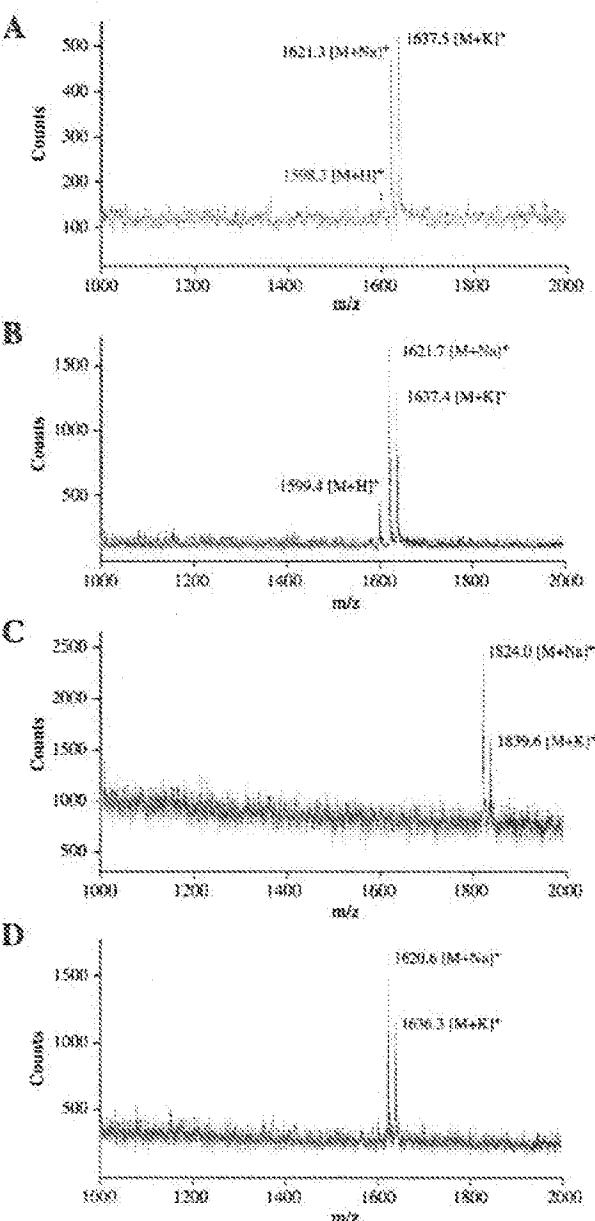


FIG. 4. MALDI-TOF MS analysis of the reaction products. MS spectra of the reaction products from GnGn-bi-PA. *A*, Gn₃-tri-PA, an enzymatic product by GnT-V as a control. *B-D*, enzymatic products P1–P3 by GnT-IX.

RESULTS

By performing a BLAST search with the amino acid sequence of human GnT-V as a query, we were able to find a human hypothetical protein that has a significant similarity to the catalytic domain of GnT-V (14), but lacking most of the deduced N-terminal region. Using the amino acid sequence of the hypothetical protein in an EST data base search, we found seven clones containing partial sequences corresponding to the hypothetical protein. From the consensus sequence of the seven EST clones, we designed oligonucleotide primers for reverse transcription-PCR using total RNA from various human tissues. Since the PCR products were strongly detected in the brain and the clone of the hypothetical protein was derived from adult brain, we used total RNA from human brain for the subsequent cDNA cloning. The 5'-end of the cDNA was determined by a combination of RACE and a further data base

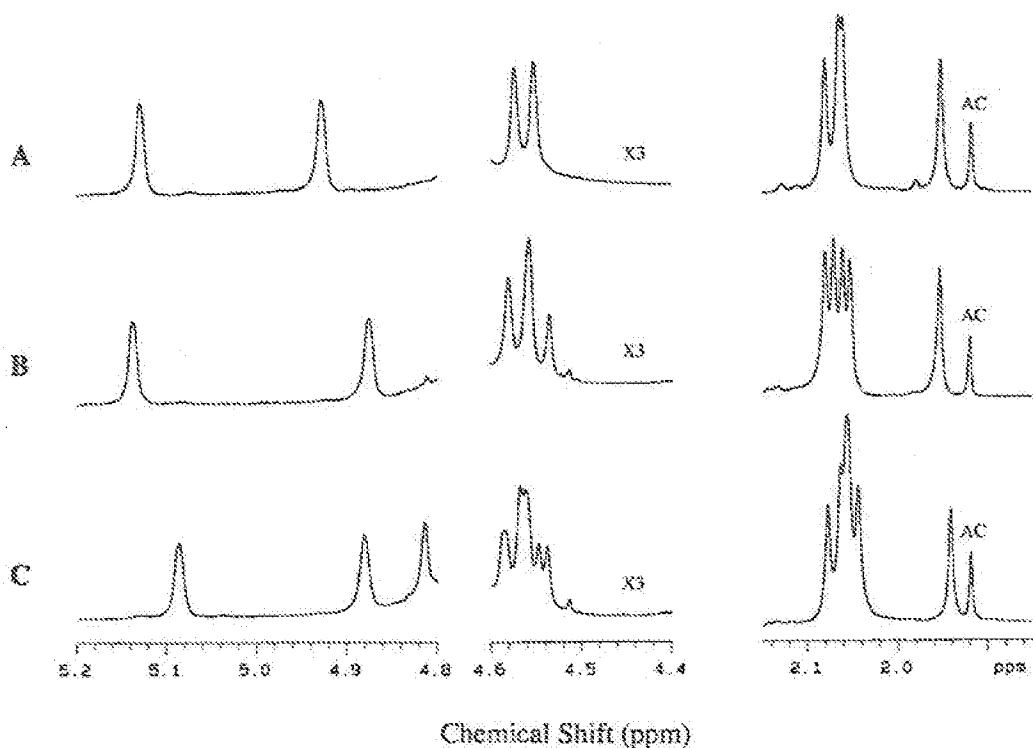


Fig. 5. Proton NMR spectra of the substrate and the enzymatic products. A, GnGn-bi-PA; B and C, enzymatic products P1 and P2. Chemical shift values of anomeric proton signals and methyl proton signals are summarized in Table I. AC indicates a signal due to ammonium acetate used as elution buffer for HPLC.

TABLE I

The chemical shifts of anomeric proton signals and methyl proton signals of the substrate GnGn-bi-PA and two enzymatic products P1 and P2

	Anomeric proton signals					
	Man 4	Man 4'	GlcNAc 5	GlcNAc 5'	GlcNAc 7	GlcNAc 7'
GnGn-bi-PA	5.137	4.926	4.566	4.566		
P1	5.136	4.875	4.571	4.573	4.549	
P2	5.086	4.879	4.578	4.575	4.552	4.561

	Methyl proton signals					
	GlcNAc 1	GlcNAc 2	GlcNAc 5	GlcNAc 5'	GlcNAc 7	GlcNAc 7'
GnGn-bi-PA	1.951	2.080	2.065	2.059		
P1	1.953	2.080	2.076	2.061	2.052	
P2	1.941	2.077	2.063	2.057	2.055	2.044

search. The resulting full-length cDNA (termed GnT-IX) and deduced amino acid sequences are shown in Fig. 1A. The deduced protein is a type II membrane protein composed of 792 amino acid residues with a calculated molecular mass of 89,531 Da and has eight potential *N*-glycosylation sites. It appears to consist of an N-terminal cytoplasmic tail, a transmembrane domain, a stem region, and a C-terminal catalytic domain, and the entire sequence identity to human GnT-V is 42%. Comparing the amino acid sequences of human GnT-V and GnT-IX with *C. elegans* ortholog GLY-2 (15) using BLAST 2 sequences (21), the sequences of human GnT-V and GnT-IX exhibit 36 and 32% identities, respectively, to the GLY-2 sequence. The human GnT-V gene (*Mga15*) is mapped to chromosome 2q21 (14), whereas the GnT-IX gene is mapped to 17q25.

Initially, we attempted to detect GnT activity using a whole cell lysate or a microsomal membrane fraction prepared from GnT-IX-transfected cells. Although we tested several cell lines, endogenous GnT-V activity was detected, and no other GnT activity was found under the conditions used in the tests. To concentrate the recombinant enzyme and to eliminate endogenous GnT-V activity, full-length human GnT-IX containing a

His₆ tag was expressed in Neuro-2a cells, captured by Ni-NTA beads, or immunoprecipitated with an anti-His tag antibody and Protein G beads, and the beads were then directly used for a subsequent GnT assay. Western blot analysis of the captured enzyme showed a 110-kDa band and a smear band at a higher molecular weight (Fig. 2). The captured tagged protein was directly subjected to GnT assay under conditions typical for GnT-V activity with GnGn-bi-PA as an acceptor substrate. When the reaction mixture was subjected to HPLC, the major product (designated as P1) of GnT-IX was eluted (peak 1 in Fig. 3A) with the same retention time as the GnT-V control (Fig. 3C). In addition, two more peaks (peaks 2 and 3) (designated as P2 and P3) that have never been seen in parental cells and GnT-V control cells were observed. These peaks were collected and subjected to MALDI-TOF MS analysis (Fig. 4). The spectrum of P1 showed an *m/z* value for (M + Na)⁺ of 1621.7 (Fig. 4B), corresponding to that of Gn₃-tri'-PA (refer to Fig. 6 for structure), 1621.3 (Fig. 4A). This result and the retention time on HPLC suggest that the structure of P1 could be same as the product of GnT-V. The spectrum of P3 (Fig. 4D) also showed nearly the same *m/z* value (1620.6) as Gn₃-tri'-PA. On the other

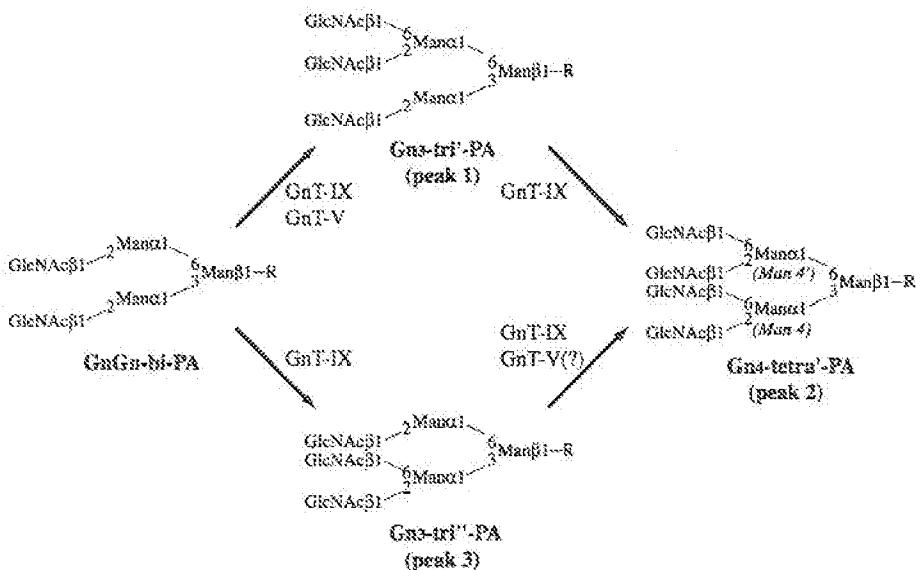


FIG. 6. Predicted synthetic pathway catalyzed by GnT-IX. GnT-IX catalyzes the transfer of GlcNAc to the both mannose arms in the core of the acceptor substrate GnGn-bi-PA via β 1,6-linkage, whereas GnT-V catalyzes transfer only to α 1,6-mannose. The structures of the acceptor substrate and the enzymatic products are shown.

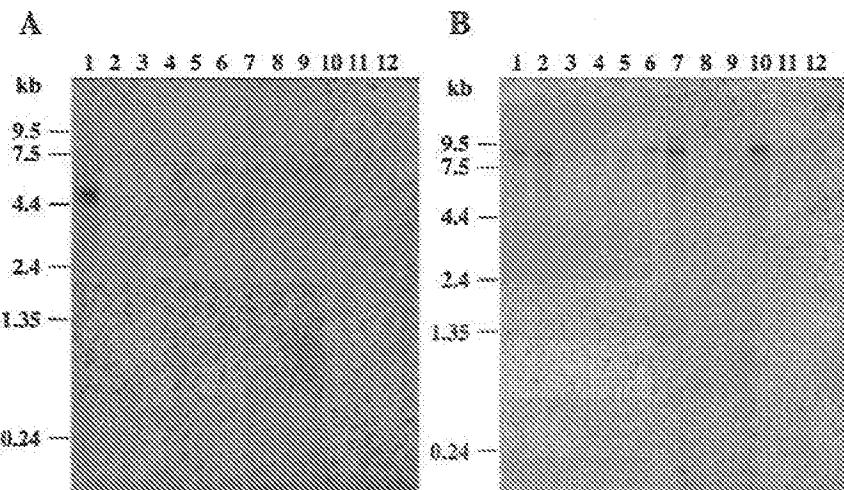


FIG. 7. Northern blot analysis of human GnT-IX and GnT-V transcripts. Human multiple tissue Northern blot was hybridized with 32 P-labeled probe for GnT-IX (A) and GnT-V (B). Lane 1, brain; lane 2, heart; lane 3, skeletal muscle; lane 4, colon (no mucosa); lane 5, thymus; lane 6, spleen; lane 7, kidney; lane 8, liver; lane 9, small intestine; lane 10, placenta; lane 11, lung; lane 12, peripheral blood leukocyte. Standards are indicated on the left.

hand, the *m/z* value of P2 was 1824.0 (Fig. 4C), indicating that two GlcNAc residues had been transferred to the acceptor substrate. Furthermore, when $\text{Gn}_3\text{-tri-PA}$ was used as a substrate, a product peak (peak 4 in Fig. 3D) appeared with the same retention time as peak 2 in Fig. 3A. Thus, GnT-IX transfers GlcNAc not only to the core α 1,6-mannose arm via a β 1,6-linkage, typical of GnT-V activity, but also to another position and/or via a distinct linkage.

Using recombinant soluble GnT-IX, the pH optimum of the GnT activity of GnT-IX was examined in a reaction mixture composed of 200 mM each buffer, 40 mM UDP-GlcNAc, 200 mM GlcNAc, 10 mM EDTA, 20 μ M GnGn-bi-PA. The optimal pH was found to be between 7.5 and 8.5 in MOPS or HEPES buffer, whereas that of GnT-V was around pH 6.5 (data not shown). The effects of divalent cations on GnT activity were also examined in the same reaction mixture with 10 mM each of metal chlorides or EDTA. Metal ions such as Mg^{2+} , Ca^{2+} , and Mn^{2+} had no effect on the reaction. GnT activity is strongly inhibited by Ni^{2+} , Cu^{2+} , and Zn^{2+} . EDTA had no effect on the activity as for GnT-V and other β 1,6GnTs. The activity of GnT-IX was enhanced by the presence of glycine. In the presence of 0.5 M glycine at pH 7.5, the yield of enzymatic products were in-

creased about 30% after a 2-h incubation (data not shown). Therefore, we carried out a large scale reaction under this condition to prepare samples for NMR analyses. After a 3-day incubation, most of the substrate was converted to the enzymatic products: 45, 39, and 7% of biantennary substrate was converted to P1, P2, and P2, respectively. Therefore, P1 and P2 were collected and subjected to NMR analyses.

NMR analyses were carried out to confirm of the structures of the enzymatic products P1 and P2. Proton NMR spectra of the substrate and the two products are shown in Fig. 5. The chemical shift values of their anomeric proton signals and methyl proton signals are summarized in Table I. The values for P1 were identical with those of $\text{Gn}_3\text{-tri-PA}$ (22). In the spectrum of the P2, a methyl proton signal of an additional GlcNAc appeared at 2.044 ppm. This chemical shift value suggests that the GlcNAc binds to the $\text{-CH}_2\text{OH}$ group at the 6-position. The anomeric proton signal of Man 4 (assigned at the $\text{Gn}_4\text{-tetra-PA}$ structure in Fig. 6) was observed at 5.086 ppm and showed a shift to higher field by 0.50 ppm relative to that of $\text{Gn}_3\text{-tri-PA}$ (spectra B and C in Fig. 6). When GlcNAc was attached to $\text{-CH}_2\text{OH}$ at the 6-position of Man 4', the anomeric proton signal of Man 4' shifted to higher field by 0.49 ppm

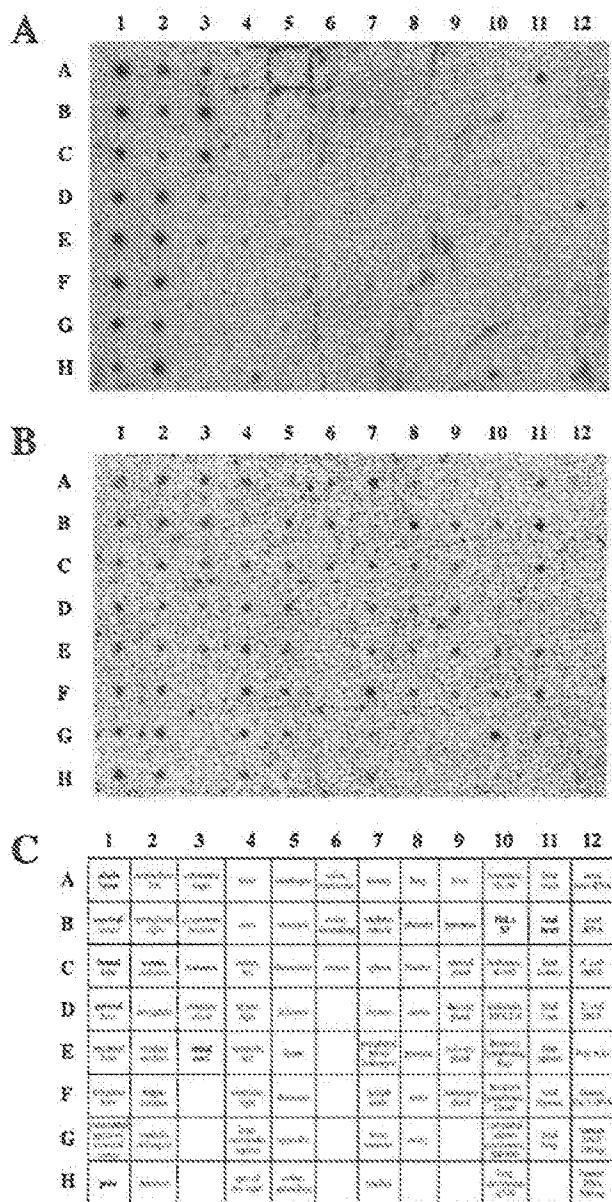


Fig. 8. Dot blot analysis of human GnT-IX and GnT-V transcripts. Human multiple tissue expression array was hybridized with ^{32}P -labeled probe for GnT-IX (A) and GnT-V (B). C, tissues of loaded poly(A)⁺ RNA are indicated.

(spectra A and B in Fig. 5). These results indicate that the additional GlcNAc of the P2 is linked to CH_2OH at the 6-position of Man 4 of the $\text{Gn}_3\text{-tri}'\text{-PA}$ (P1). The anomeric proton of the additional GlcNAc (GlcNAc 7') gave a doublet signal with a coupling constant of 8.0 Hz at 4.561 ppm. This coupling constant value shows that the linkage of the GlcNAc is a β -form.

Northern blot analysis showed that the GnT-IX transcript of ~ 5 kb is exclusively expressed in the brain, whereas the GnT-V transcript of ~ 9 kb is expressed in various tissues (Fig. 7). Dot blot analysis also showed that GnT-IX is specifically expressed in all areas of the adult brain as well as the fetal brain (Fig. 8).

DISCUSSION

Thus far, no homologous gene of GnT-V in the genome in any mammalian species has been reported. Recently, a functional GnT-V ortholog was found in *C. elegans*, and the gene was the sole GnT-V homolog present in its genome (15). Here we report

a novel homologous gene of human GnT-V, designated as GnT-IX, which possesses novel β 1,6GnT activity.

The domain architecture and the positions of the cysteine residues of mammalian and nematode GnT-Vs are well conserved in GnT-IX. In addition, Leu¹⁸⁹ in the stem region of human GnT-V, corresponding to the position at a L188R point mutation in Lec4A Chinese hamster ovary mutant cells (23), is also conserved in GnT-IX (Leu²⁸¹). It has previously been reported that the leucine residue is responsible for the Golgi localization of hamster and nematode GnT-Vs (15, 23). Therefore, GnT-IX is structurally related to GnT-V and may also be localized in the Golgi apparatus. Compared with GnT-V, GnT-IX has an extra 38 amino acid residues inserted in the C-terminal catalytic domain (Fig. 1B). Since two potential *N*-glycosylation sites exist in this short sequence, it may be exposed to the molecular surface. Whether this sequence is involved in the difference of substrate specificity between GnT-IX and GnT-V remains to be investigated.

Recombinant human GnT-IX actually exhibited GnT-V activity, catalyzing the transfer of GlcNAc to the 6-OH position of the α 1,6-linked mannose arm of $\text{Gn}_3\text{-bi}'\text{-PA}$ and forming $\text{Gn}_3\text{-tri}'\text{-PA}$ (Fig. 6). This activity of GnT-IX was considerably lower than that of GnT-V when a similar amount of expression was observed as judged by reactivity to an anti-His tag antibody (data not shown). Interestingly, GnT-IX also acted on the α 1,3-linked mannose arm in the acceptor substrate. Therefore, GnT-IX is able to catalyze the transfer of GlcNAc to the 6-OH position of mannose in the sequence GlcNAc β 1,2-Man1, which is present in both α -linked mannose arms of the *N*-glycan, indicating that GnT-IX is different and distinct from GnT-V. GnT-IX preferentially transferred GlcNAc to the α 1,6-linked mannose arm rather than the α 1,3-linked mannose arm (peaks 1 and 3 in Fig. 3A), suggesting that the 6-OH position of α 1,6-linked mannose is more accessible to GnT-IX than that of the α 1,3-linked mannose.

The expression of the GnT-IX gene was found to be exclusively in the brain, whereas the GnT-V gene is ubiquitously expressed. Similar results were also observed in fetal tissues, suggesting that GnT-IX may play important roles in fetal and adult brains. In addition, although *Mgat5*^{-/-} mice lacked most detectable GnT-V enzyme activity and *P. vulgaris* leukoagglutinin reactivity, brain development and function in these mice appeared to be normal (12). Since the poly-*N*-acetyllactosamine extension is dependent on β 1,6-branch formation by GnT-V, β 1,6-branch formation is thought to be important in the process of nerve differentiation via the interaction of poly-*N*-acetyllactosamine chains and their receptors (4, 5). GnT-IX may compensate for the deficiency in *Mgat5*^{-/-} brain with regard to neuronal development and functions.

Fig. 6 summarizes the predicted synthetic pathway of the core structure in *N*-glycan catalyzed by GnT-IX. GnT-IX catalyzes the synthesis of two unique *N*-linked oligosaccharide structures, namely the triantennary oligosaccharide possessing a β 1,6-branched GlcNAc on the α 1,3-linked mannose arm ($\text{Gn}_3\text{-tri}'\text{-PA}$, peak 3) and the tetraantennary oligosaccharide containing two β 1,6-branched GlcNAc residues in the core mannose ($\text{Gn}_4\text{-tetra}'\text{-PA}$, peak 2). These structures have never been reported in any mammalian tissues. For further progress, it will be necessary to determine whether these structures actually exist in the brain.

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Origin of the protein						
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	Synonyms	GnT-IX		
From	Homo sapiens (Human)[TaxID:9606]			
Taxonomy	Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Primates; Haplorhini; Catarrhini; Hominidae; Homo.			
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<p>[1] NUCLEOTIDE SEQUENCE. TISSUE=Brain; DOI=10.1074/jbc.M308255200; MEDLINE=22940126; PubMed=12941944; [NCBI, ExPASy, EBI, Israel, Japan] Inamori K., Ende T., Ida Y., Fujii S., Gu J., Honke K., Taniguchi N.; "Molecular cloning and characterization of human GnT-IX, a novel beta1,6-N-acetylglucosaminyltransferase that is specifically expressed in the brain."; <i>J. Biol. Chem.</i> 278:43192-43199(2003).</p> <p>[2] NUCLEOTIDE SEQUENCE. TISSUE=Brain; Inamori K., Taniguchi N.; Submitted (APR-2003) to the EMBL/GenBank/DBJ databases.</p>				
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Cross-references				
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HGNC	HGNC:24140; MGAT5B.			
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